

THE SYNTHESIS OF  
POLYMER-BOUND ADENINE NUCLEOTIDES  
AND THEIR USE AS  
AFFINITY ADSORBENTS AND ACTIVE COFACTORS

BY

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## CONTENTS

|                                                                                                |    |
|------------------------------------------------------------------------------------------------|----|
| Publications .....                                                                             | 3  |
| Introduction .....                                                                             | 4  |
| General considerations relating to the preparation of immobilized<br>adenine nucleotides ..... | 5  |
| The point of attachment .....                                                                  | 5  |
| The extension arm .....                                                                        | 6  |
| The polymer support .....                                                                      | 6  |
| Direct versus indirect attachment .....                                                        | 8  |
| Adenine nucleotide derivatives suitable for immobilization .....                               | 10 |
| N <sup>6</sup> alkyl substituted nucleotides .....                                             | 10 |
| N <sup>6</sup> acyl substituted nucleotides .....                                              | 17 |
| S <sup>6</sup> alkyl substituted nucleotides .....                                             | 18 |
| C8 substituted nucleotides .....                                                               | 18 |
| Phosphate substituted nucleotides .....                                                        | 19 |
| Ribose substituted nucleotides .....                                                           | 20 |
| General discussion .....                                                                       | 22 |
| Acknowledgements .....                                                                         | 24 |
| References .....                                                                               | 25 |





## PUBLICATIONS

This thesis is based on the following papers, which are referred to by Roman numerals in the text.

- I. On Adenine Nucleotides for Affinity Chromatography.  
Guilford, H., Larsson, P.O. and Mosbach, K. (1972)  
Chemica Scripta 2, 165-170.
- II. The Synthesis of Three AMP-Analogues: N<sup>6</sup>-(6-Aminohexyl)-adenosine 5'-Monophosphate, N<sup>6</sup>-(6-Aminohexyl)-adenosine 2',5'-Bisphosphate, and N<sup>6</sup>-(6-Aminohexyl)-adenosine 3',5'-Bisphosphate and Their Application as General Ligands in Biospecific Affinity Chromatography.  
Brodelius, P., Larsson, P.O. and Mosbach, K. (1974)  
Eur. J. Biochem. 47, 81-89.
- III. A New Immobilized NAD<sup>+</sup> Analogue, Its Application in Affinity Chromatography and as a Functioning Coenzyme.  
Lindberg, M., Larsson, P.O. and Mosbach, K. (1973)  
Eur. J. Biochem. 40, 187-193.
- IV. Preparation of a NAD(H)-Polymer Matrix Showing Coenzymic Function of the Bound Pyridine Nucleotide.  
Larsson, P.O. and Mosbach, K. (1971)  
Biotechnol. Bioeng. 13, 393-398.
- V. The Preparation and Characterisation of a Water-Soluble Coenzymically Active Dextran-NAD<sup>+</sup>.  
Larsson, P.O. and Mosbach, K. (1974)  
FEBS Lett. 46, 119-122.





## INTRODUCTION

The rapid development of methods for the preparation of immobilized, i.e. polymer-bound, nucleotides closely parallels the spectacular success of affinity chromatography. Affinity chromatography exploits the specific interaction between a biological macromolecule and its polymer-bound substrate, inhibitor, cofactor or effector to achieve separation and thus differs fundamentally from traditional methods of separation, which largely depend on rather non-specific physico-chemical parameters such as size and charge. The first reports on affinity chromatography dealt almost exclusively with ligands specific for one particular enzyme (1-3). Very convincing purifications were usually achieved, although a decided disadvantage seemed the fact that a unique affinity adsorbent had to be prepared and evaluated for each new enzyme under study. More versatile affinity adsorbents showing specificity towards groups of enzymes seemed a promising alternative and the concept of "general ligand affinity chromatography" soon attracted much attention (4-5). The development of general ligand chromatography became closely dependent on the successful preparation of various immobilized adenine nucleotides, since as expected these derivatives proved to be excellent general ligands for classes of enzymes such as kinases and  $\text{NAD}^+$ - and  $\text{NADP}^+$ -dependent dehydrogenases. Several immobilized adenine nucleotides have been synthesized and derivatives with many of the ideal characteristics for a particular separation problem are now available.

Recent trends in enzyme technology have also considerably stimulated the interest in polymer-bound adenine nucleotides. Several technologically interesting enzymes require cofactors such as  $\text{NAD}^+$  or  $\text{NADP}^+$  for their catalytic activity and to allow an economic exploitation of these enzymes, the expensive cofactor must be re-used. Binding to a soluble high molecular weight support provides a method of retaining the cofactor within an enzyme reactor and may thus render sophisticated processes like enzymic steroid conversion feasible (6). The potential use of cofactors in medicine and in analytical applications has further promoted the interest in polymer-bound adenine nucleotides.





## GENERAL CONSIDERATIONS RELATING TO THE PREPARATION OF IMMOBILIZED ADENINE NUCLEOTIDES

### The point of attachment

The crucial question underlying the preparation of a new polymer-bound ligand is where the necessary substitution may be performed without loss of the biological activity towards the complementary enzyme or class of enzymes. Data concerning the biological properties of analogues of the ligand give valuable guidance and, if available, the structure of the enzyme - ligand complex as revealed by high resolution x-ray crystallography may provide further information. Solvent-exposed parts of the ligand would be the most likely candidates for successful substitution.

Adenine nucleotides have been immobilized through positions C8 and N<sup>6</sup> of the adenine ring and through the ribose hydroxyls and phosphate moieties and used as ligands for dehydrogenases and kinases with varying success. There is a pronounced tertiary structure homology in the cofactor binding site of several dehydrogenases. When the cofactor is bound to the active site of dehydrogenases both positions N<sup>6</sup> and C8 of the adenine ring are fairly accessible to solvent and are not involved in binding of the cofactor to the enzyme (7). In contrast, the ribose hydroxyls are buried in the crevice and hydrogen bonded to amino acid side chains. Thus, dehydrogenases usually interact efficiently with nucleotides immobilized through positions N<sup>6</sup> and C8 but exhibit little binding to ribose-substituted ligands. It must, however, be emphasized that it is the architecture of the individual enzyme which will determine if a particular substitution is compatible with retained biological activity or not. Conversely, enzymes showing very similar chromatographic behaviour might be expected to have related structural properties of their active sites.

Attachment through the nicotinamide moiety of cofactors such as NAD<sup>+</sup> has not been reported. Several soluble NAD<sup>+</sup>-analogues bearing a modified nicotinamide entity are, however, known to be coenzymically active (8).





### The extension arm

An important feature of the polymer-bound nucleotide is the spacer or the extension arm. Early reports on affinity chromatography showed that more efficient adsorbents were achieved if the ligand was attached to the polymer via an extension arm, such that shielding of the ligand by the polymer backbone could be annulled and permit proper contact with the enzymes (2). Most immobilized adenine nucleotides have also been provided with a "hexamethylene" spacer, and recent systematic investigations have indeed proved the value of such an entity (9,10). The minimum length required of the extension arm is dependent on the morphology of the individual enzyme, but the ubiquitously used hexamethylene spacer appears to provide at least near-optimum conditions for most enzymes.

In some aspects the hexamethylene spacer is not ideal. Its rather lipophilic character might lead to non-specific interaction with hydrophobic sites on protein surfaces and thereby complicate the separation. The existence of such hydrophobic interactions in chromatographic processes has been convincingly demonstrated in a technique termed "hydrophobic chromatography", where the chromatographic material contains aliphatic hydrocarbon chains, which quite firmly adsorb several proteins (11). If the hydrophobic region of an enzyme is situated in close proximity to the binding site of the ligand, a rather specific enhancement of the affinity might occur. Enzymes with comparatively low affinity towards a free ligand might of this reason be efficiently and specifically adsorbed to the support - spacer - ligand assembly. An example of this effect might be lactate dehydrogenase whose complex with gel-bound  $N^6$ -(6-aminoethyl)-AMP has been measured to be several orders of magnitude stronger than the complex with free ligand (12).

### The polymer support

Supports for affinity chromatography must meet several requirements to allow efficient ligand - enzyme interaction. The structure must be sufficiently porous to allow unimpeded passage of macromolecules, but still have enough mechanical stability to withstand column operations without compression. To minimize non-specific interaction the matrix



material should be hydrophilic and not contain appreciable amounts of ionic sites. Moreover, it should be possible to covalently attach the ligand whilst retaining these properties.

Sepharose fulfills these requirements fairly well and consequently is extensively used in combination with the cyanogen bromide coupling method (13). The coupling of amino compounds to cyanogen bromide activated Sepharose is thought to result mainly in protonated N-substituted isoureas which carry a positive charge at physiological pH-values. Ionic species are thus introduced with this otherwise excellent method, although charge-free linkages may be obtained by the use of ligands carrying a terminal hydrazide group (14). Beaded polyacrylamide and porous glass have also been used as supports for adenine nucleotides although not as frequently as Sepharose.

Somewhat different properties are required of the polymer support if it is designed to carry an active nucleotide cofactor such as  $\text{NAD}^+$ . The need for fast and efficient interaction between ligand and enzyme becomes a dominating feature. Soluble high molecular weight supports with a loose three-dimensional structure are suitable, since they are not only easily confined by semi-permeable membranes, but are also orders of magnitude more efficient than solid supports. In paper V this is clearly demonstrated. Sepharose-bound  $\text{NAD}^+$  and dextran-bound  $\text{NAD}^+$  were compared as cofactors for alcohol dehydrogenases and lactate dehydrogenase, and the soluble dextran-bound  $\text{NAD}^+$  proved to be 20 - 100 times more efficient than  $\text{NAD}^+$  bound to a solid support. Within the solid support the diffusion rate of the enzyme will decrease and the severely restricted mobility imposed on the bound cofactor will slow down and even prevent its interaction with the enzyme. Furthermore, after the catalytic event, the enzyme molecule might be trapped in a binary complex with the (reduced) cofactor and since adjacent cofactors on the matrix are normally too far away to interact with the complex, it could have a considerable lifetime and influence the conversion rate. Exactly the same situation prevails during the adsorption stage of affinity chromatography, where binary complex formation forms the very basis of the technique. In the case of immobilized cofactors, however, the trapping is far from desirable, since it effectively





lowers the concentration of catalytically operative enzyme. Intrinsically soluble carriers would be almost devoid of these rate-limiting factors and would thus allow continuous and efficient interaction between bound cofactor and enzyme.

Soluble dextran appears to be a suitable carrier for active cofactors and its application in enzyme reactors and enzyme electrodes substantiates this (15). Soluble polyethyleneimine has also been used as a carrier for active cofactors and may even be preferable when carboxyl-containing cofactor analogues are immobilized. Polyethyleneimine bears an abundance of charged primary, secondary and tertiary amino groups and would be considered totally unsuitable for affinity chromatography although as a carrier for coenzymically active  $\text{NAD}^+$  it has been shown to be useful (16). One obvious disadvantage which polyethyleneimine shares with all heavily charged carriers is the poor solubility in media which contain bi- or polyvalent counter-ions. The charged matrix might also interact adversely with some enzymes.

#### Direct versus indirect attachment

The easiest way of preparing a polymer-bound nucleotide would be to condense the parent nucleotide with a functional group which is already attached to the polymer via an extension arm. This has indeed been realized (IV,9,17) and such derivatives are useful for many applications. There are, however, some inherent disadvantages with this otherwise attractive direct procedure. The preparation will undoubtedly contain an excess of functional groups which will interact non-specifically with proteins and thus will usually diminish the resolving power of the chromatographic material. Furthermore, adenine nucleotides have several potentially reactive sites and the binding to a functionalized polymer may thus yield heterogeneous adsorbents containing a plethora of chemically non-equivalent ligands that might interact differently with a given enzyme.

Similar problems may also be experienced when solid phase "modular assembly" of dinucleotides, for example, are attempted (17). When polymer-bound AMP is condensed with added free NMN polymer-bound  $\text{NAD}^+$  is obtained. The procedure is relatively easy, but the resulting





adsorbent will, however, besides the desired polymer-bound  $\text{NAD}^+$  contain varying amounts of bound AMP and NMN, which might interact adversely and, in some cases, obscure otherwise clean-cut separation patterns.

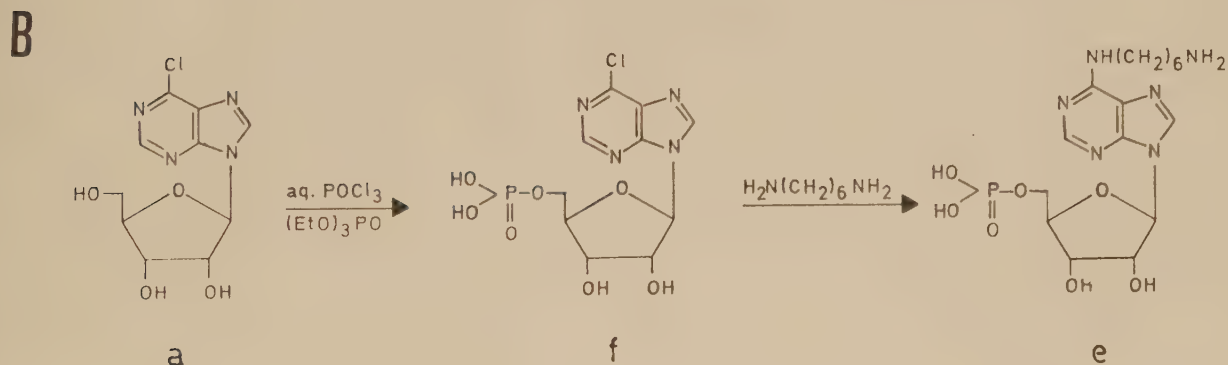
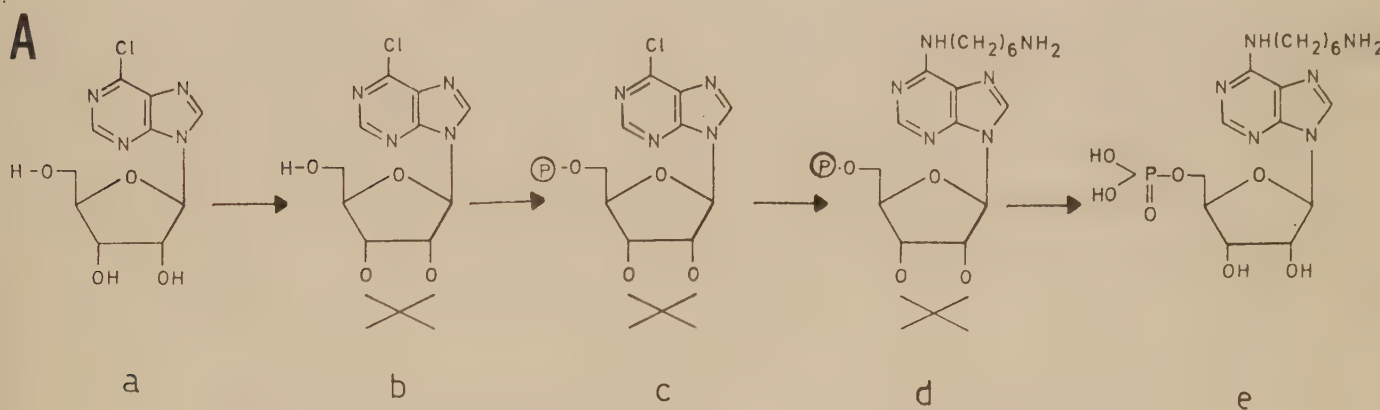
The other principal way of preparing polymer-bound nucleotides is usually considerably more elaborate, time-consuming and costly but yields products lacking the above limitations. In this approach the nucleotide is converted into an analogue carrying an extension arm with a terminal reactive functional group. This pre-assembled analogue is then coupled to the polymer. The procedure permits a careful purification of the intermediate nucleotide - spacer arm assembly and as the functional group is usually an amino group, coupling to the polymer (Sepharose) is facilitated with the cyanogen bromide method that introduces only a small proportion of ionic sites on the polymer. This last approach, giving well-characterized polymer derivatives (I,II,III, 18-20), must be considered the only suitable one when more precise information of the interaction between enzyme and ligand is desired.



# ADENINE NUCLEOTIDE DERIVATIVES SUITABLE FOR IMMOBILIZATION

## N<sup>6</sup> alkyl substituted nucleotides

The first reported nucleotide of this type suitable for attachment to polymers was N<sup>6</sup>-(6-aminohexyl)-adenosine 5'-monophosphate, prepared by displacing the chloro group of 6-chloropurine riboside phosphate with 1,6-diaminohexane (I). The same compound has also been prepared from 6-mercaptapurine riboside phosphate in an analogous fashion (18). In paper I two alternative routes are given for the synthesis of N<sup>6</sup>-(6-aminohexyl)-AMP:



a = 6-chloropurine riboside

b = 6-chloropurine 2',3'-O-isopropylideneriboside

c = 6-chloropurine 2',3'-O-isopropylideneriboside phosphate

d = N<sup>6</sup>-(6-aminohexyl)-adenine 2',3'-O-isopropylideneriboside phosphate

e = N<sup>6</sup>-(6-aminohexyl)-adenosine 5'-monophosphate

f = 6-chloropurine riboside phosphate





In route "B" the phosphorylating agent phosphoryl chloride is mixed with a small amount of water to suppress the formation of 2'/3' phosphorylated nucleotides, and thus facilitates a shorter route without the elaborate and tedious protection and deprotection of the secondary ribose hydroxyls. The last step in the synthesis, i.e. the displacement of the chloro group with 1,6-diaminohexane, was originally carried out by heating the 6-chloropurine riboside phosphate for 6 hours at 80° C with a 30-fold molar excess of neutralized diamine. Later investigations, however, have shown that a moderate five- to ten-fold excess is quite satisfactory to prevent the formation of any appreciable amount of disubstituted diamine, provided the pH is kept at about 11. The unprotonated diamine then reacts smoothly with the chloropurine compound and the displacement is completed within one hour at room temperature. Side reactions, e.g. hydrolysis into inosine monophosphate, are barely detectable. A more convenient procedure for the preparation of N<sup>6</sup>-(6-aminohexyl)-AMP has thus become available and the essential features are given below (not reported elsewhere):

Chloropurine riboside (20 mmol, 5.7 g) is suspended in 20 ml of triethyl phosphate and the phosphorylating agent (60 mmol POCl<sub>3</sub> and 20 mmol H<sub>2</sub>O in 2.5 ml triethyl phosphate) is added in three portions over a period of one hour. After two hours at 0° C the conversion is complete as judged by thin layer chromatography and the product is precipitated with a mixture of diethyl ether and petroleum ether (1:1, v/v). The precipitate is transferred in portions to ice-water, whose pH is kept at 8 by addition of LiOH from an automatic titrator. The yield of 6-chloropurine riboside 5'-phosphate is approximately 85 % as judged by ultra-violet measurements.

Hexamethylenediamine (50 - 100 mmol) is added to 50 - 100 ml of an aqueous solution of 6-chloropurine riboside phosphate (10 mmol) and after 1 h at room temperature the conversion into N<sup>6</sup>-alkylated nucleotide is complete. Ion exchange chromatography on Dowex 1X-8 (acetate) yields N<sup>6</sup>-(6-aminohexyl)-AMP in an overall yield of about 75 %.

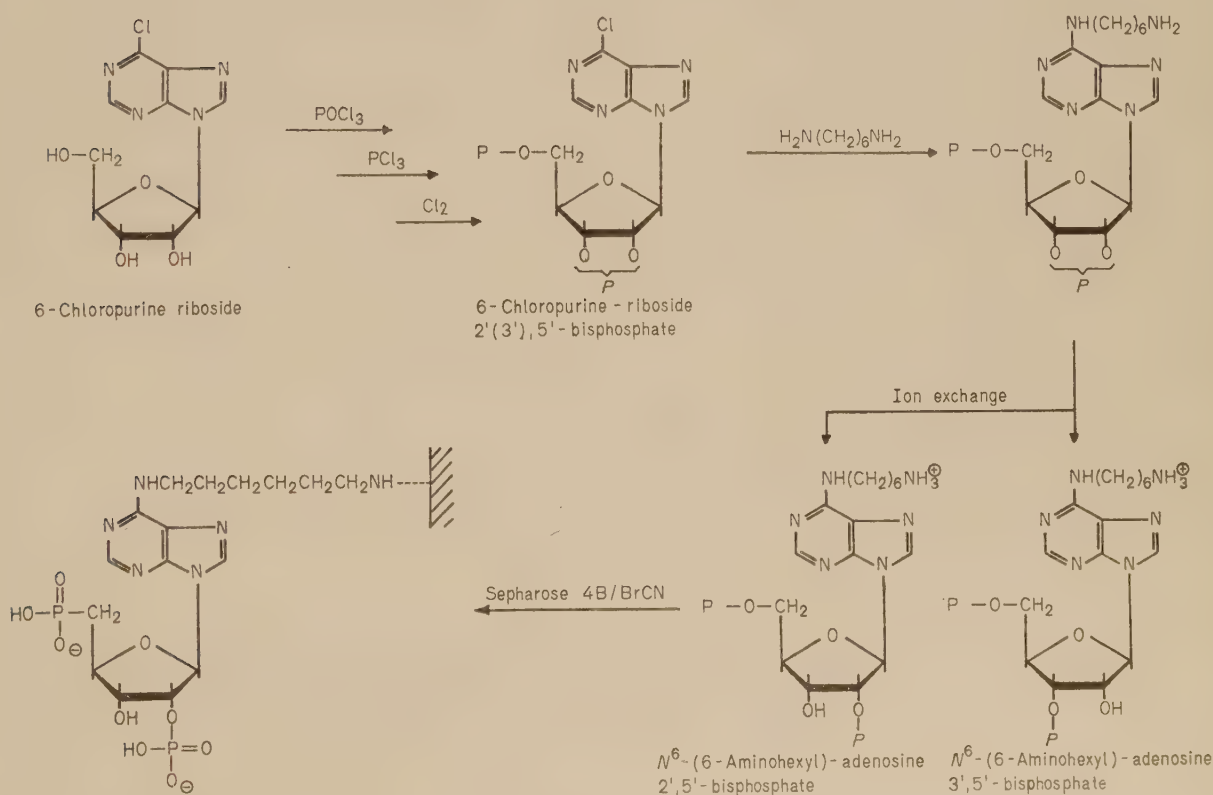




This simplified procedure has made it possible to synthesize  $N^6$ -(6-aminohexyl)-AMP in large amounts at a reasonable cost and the compound will soon be commercially available in Sepharose-bound form (Pharmacia Fine Chemicals).

$N^6$ -(6-aminohexyl)-AMP may be attached to carriers such as Sepharose and dextran by the cyanogen bromide method and the polymer-bound derivative has proven versatile in numerous applications. It exhibits good affinity properties towards a number of kinases and  $NAD^+$ -dependent dehydrogenases (12,18,19,21-23) as shown by model studies and successful purifications from crude extracts. It has also been used as an immobilized effector for glycogen phosphorylase b (24) and as a tool for rapid estimation of  $K_{diss}$ -values (25).

The method of synthesis described above formed a suitable basis for the preparation of  $N^6$ -(6-aminohexyl)-adenosine 2'(3'),5'-bisphosphate (II):





The phosphorylating agents employed in this synthesis are phosphoryl chloride and phosphorus trichloride. Phosphoryl chloride reacts readily with the primary ribose hydroxyl (5') but slowly and incompletely with secondary ones (2'/3'). Phosphorus trichloride on the other hand is reasonably reactive with secondary hydroxyls whilst still being very reactive with primary ones. Unfortunately, phosphorus trichloride cannot be used for the simultaneous phosphorylation of both sites, since the 5'-phosphorodichloridite intermediate will decompose to a large extent before the bulk of 2'/3' hydroxyls are phosphorylated (26). By using both reagents in two consecutive steps and then oxidizing the 2'(3')-phosphorochloridite with gaseous chlorine into 2'(3')-phosphorochloridate, a bis-phosphorylated nucleoside is obtained in good yield.

Sepharose-bound  $N^6$ -(6-aminohexyl)-adenosine 5'-monophosphate, Sepharose-bound  $N^6$ -(6-aminohexyl)-adenosine 2',5'-bisphosphate and Sepharose-bound  $N^6$ -(6-aminohexyl)-adenosine 3',5'-bisphosphate were compared as affinity adsorbents for several  $NAD^+$ - and  $NADP^+$ -dependent dehydrogenases (II). The first derivative would be expected to retain  $NAD^+$ -dependent enzymes and the second  $NADP^+$ -dependent ones. The third derivative, i.e. the 3',5'-bisphosphate, was utilized as the "ideal blank" adsorbent and would be expected to show no appreciable affinity towards either of the two types of dehydrogenases. The anticipated results were in fact obtained, demonstrating a well expressed specificity of the respective enzyme for its complementary gel-bound ligand. Some exceptions were noted however. The  $NADP^+$ -dependent glucose-6-phosphate dehydrogenase (*Candida utilis*) was not only adsorbed to the Sepharose-bound 2',5'-analogue but also to the other two materials. A satisfactory explanation for this behaviour was readily obtained from enzyme inhibition studies, since the glucose-6-phosphate dehydrogenase was significantly inhibited by both the 5'- and the 3',5'-analogue.

$N^6$ -(6-aminohexyl)-adenosine 3',5'-bisphosphate might be an interesting ligand for CoA-dependent enzymes, provided the substitution in  $N^6$  does not adversely interfere in the binding processes.

$N^6$ -substituted analogues of ADP and ATP are readily prepared from  $N^6$ -(6-aminohexyl)-AMP (19). The procedure involves an initial protection

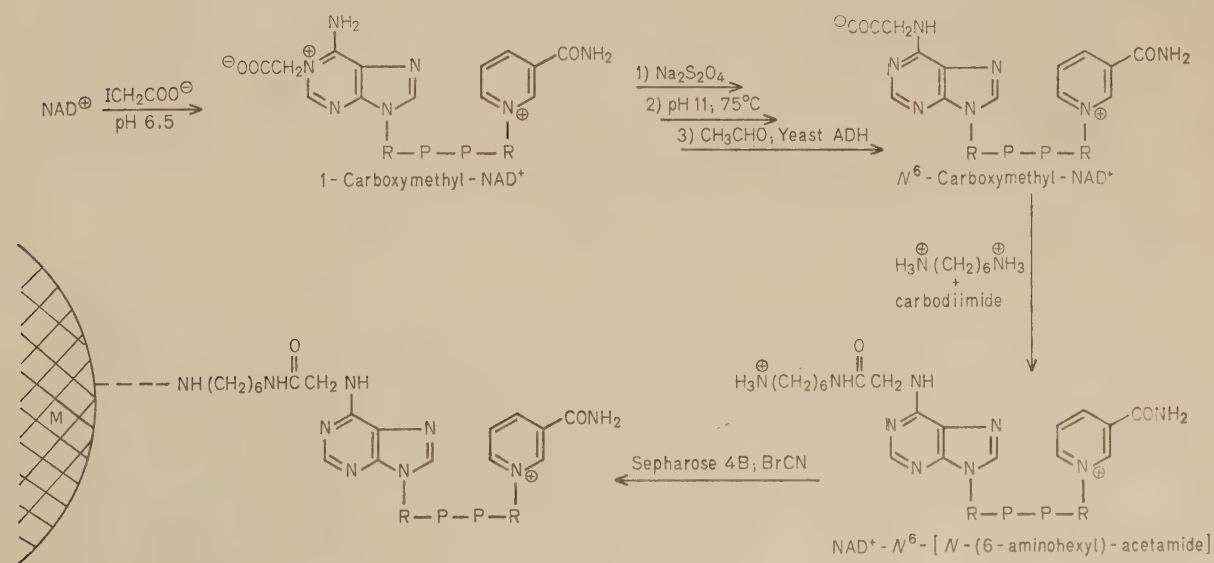




of the amino group with a trifluoroacetyl function. Subsequent treatment with carbonyl diimidazole gives a nucleotide imidazolide, which is allowed to react with phosphate or pyrophosphate. The resulting nucleoside (di-) triphosphate is finally deprotected in alkali.

The synthesis of the corresponding analogues of  $\text{NAD}^+$  and  $\text{NADP}^+$  is possible by this technique, i.e. condensation of an AMP- or ADP-analogue with NMN. There are however some inconvenient aspects associated with the method. If the condensation is promoted by agents such as carbodiimides or trifluoroacetic acid anhydride, several side reactions will diminish the yield of the desired compound, and if agents like carbonyl diimidazole are utilized stringent demands on dry conditions are essential for reasonable yields. Good yields are essential, not only because subsequent purifications are easier but mainly due to the very high cost of NMN. Nevertheless the method has been successfully adopted in small-scale preparations and the resulting  $\text{N}^6$ -(6-aminohexyl)- $\text{NAD}^+$  has exhibited the expected properties in the affinity chromatography of  $\text{NAD}^+$ -dependent dehydrogenases (27). Needless to say, it should also prove to be a suitable compound for studies of immobilized active cofactors.

In this laboratory efforts to prepare immobilized  $\text{NAD}^+$  were concentrated on methods that allowed direct introduction of the required spacer into the native  $\text{NAD}^+$  molecule. In paper III such a procedure is presented and the route of synthesis is depicted below:





The synthesis involves a rearrangement step where 1-carboxymethyl-NADH is converted into 6-carboxymethyl-NADH by heating (75° C) the 1-substituted analogue for one hour at pH 11. The rearrangement step must be carried out with the reduced nucleotide, since oxidized nucleotides are labile in alkaline medium. The Dimroth-type rearrangement proceeds via ring-opening, reorientation and a final recyclization. The validity of this mechanism has been established by chemical methods (28) and recently also by elegant use of mass spectrometry (29).

Recent studies of the cofactor properties of polymer-bound NAD<sup>+</sup>-analogue (V,15) made it necessary to prepare N<sup>6</sup>-carboxymethyl-NAD<sup>+</sup> and NAD<sup>+</sup>-N<sup>6</sup>-[N-(6-aminoethyl)-acetamide] in rather large quantities and it thus seemed worthwhile to improve the original procedure with respect to yield and reaction time. The example given below differs from the published procedure in some details and is characterized mainly by the use of higher concentrations of iodoacetic acid, a slightly different procedure to obtain the reduced compound and more carefully controlled conditions for attaching the hexamethylene spacer. Furthermore, somewhat trivial details as modified precipitation methods are quite beneficial for large-scale preparations.

#### N<sup>6</sup>-carboxymethyl-NAD<sup>+</sup>

NAD<sup>+</sup> (10 g, 13.6 mmol) is added to an aqueous solution containing 30 g iodoacetic acid (160 mmol) neutralized with LiOH, and the pH of the mixture adjusted to 6.5 with 2 M LiOH. The solution (total volume 35 ml) is kept in the dark at room temperature for 5 to 6 days and the pH intermittently adjusted to 6.5 with 2 M LiOH. The pH is then lowered to 3.0 with 6 M HCl, two volumes of ethanol added and the solution poured into 1.5 l of vigorously stirred ethanol (0° C). The precipitated crude 1-carboxymethyl-NAD<sup>+</sup> is filtered off and washed with ethanol and ether and dried in a vacuum.

The crude 1-carboxymethyl-NAD<sup>+</sup> is dissolved in 300 ml 2 % NaHCO<sub>3</sub>, pH 8.5, and the solution de-aerated with 95 % N<sub>2</sub> - 5 % CO<sub>2</sub>. The reducing agent sodium dithionite (5 g) is added and after 2 h at room temperature in the dark, the reduction is terminated by





oxygenation for 10 min followed by a brief treatment with nitrogen gas.

The reduced compound is subsequently rearranged to N<sup>6</sup>-carboxymethyl-NADH by adjusting the pH of the solution to 11.5 with NaOH and heating (75<sup>0</sup> C) for 1 h.

Finally an enzymatic reoxidation step is undertaken by adding the following: 20 ml 2 M Tris, 5 ml redistilled acetaldehyde, 3 M HCl to a final pH of 7.5 and 5 mg yeast ADH (2 500 U). After 30 min at room temperature the oxidation is complete as judged by spectrophotometric measurements and the pH is lowered to 3.5 and the crude N<sup>6</sup>-carboxymethyl-NAD<sup>+</sup> precipitated with ethanol.

The crude product is purified by ion exchange chromatography on Dowex AG 1X-2 (200 - 400 mesh, chloride). The column (2.5 x 75 cm) is loaded with the dissolved crude substance, washed with water and 0.005 M CaCl<sub>2</sub>, pH 2.7, until the pH of the effluent is about 2.8. A linear gradient, 0.005 M CaCl<sub>2</sub>, pH 2.7 to 0.05 M CaCl<sub>2</sub>, pH 2.0, (total volume 8 l) is applied. The effluent between approximately 3.3 l and 5.2 l contains the desired compound. Concentration on a rotary evaporator and precipitation with ethanol gives N<sup>6</sup>-carboxymethyl-NAD<sup>+</sup> in a yield of 40 - 45 % (5.6 g).

NAD<sup>+</sup>-N<sup>6</sup>-[N-(6-aminohexyl)-acetamide]

N<sup>6</sup>-carboxymethyl-NAD<sup>+</sup> (4.5 g, 5 mmol) is dissolved in 60 ml of 2 M 1,6-diaminohexane dihydrochloride and the solution kept in an ice-bath. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (1.4 g, 7.5 mmol) dissolved in 5 ml water is added and the pH kept at 4.8 by adding 1 M HCl or 1 M LiOH. After 10 min, when the reaction is slowing down, the ice-bath is removed and the condensation is allowed to proceed for further 45 min at room temperature. The pH is then increased to 6.5 and one volume of 0.5 M LiCl in ethanol is added and the resulting precipitate filtered off and discarded. The supernatant is slowly poured into a five-fold volume of ethanol and the precipitated crude NAD<sup>+</sup>-N<sup>6</sup>-[N-(6-aminohexyl)-acetamide] collected. The crude compound is dissolved in 75 ml



of water (pH 8) and the solution passed through a column containing 10 g of lithium-charged Dowex 50W-X4 and then through a column packed with 10 g of chloride-charged Dowex 1-X4. The final effluent is adjusted to pH 6.8 and concentrated to about 25 ml. Precipitation with ethanol gives pure  $\text{NAD}^+ - \text{N}^6 - [\text{N} - (6\text{-aminohexyl}) - \text{acetamide}]$  in a yield of about 80 % (3.3 g), the overall yield from  $\text{NAD}^+$  being approximately 35 %.

The intermediate  $\text{N}^6$ -carboxymethyl- $\text{NAD}^+$  is obviously suitable for attachment to carriers functionalized with amino groups. Consequently,  $\text{N}^6$ -carboxymethyl- $\text{NAD}^+$  was bound to water-soluble polyethyleneimine and to water-soluble aminohexyl-dextran, using carbodiimide as condensing agent, and in both cases derivatives with high nucleotide contents were obtained (30). These derivatives were, however, not particularly well suited for active cofactor studies. The activity of yeast alcohol dehydrogenase was for example strongly inhibited by the charged carriers and in some cases even enzyme denaturation occurred. It seemed a better choice to prepare the amino-containing analogue  $\text{NAD}^+ - \text{N}^6 - [\text{N} - (6\text{-aminohexyl}) - \text{acetamide}]$  and attach it to cyanogen bromide activated soluble dextran. These latter preparations proved to be superior and were successfully adopted in cofactor applications (V,15).

The Sepharose-bound  $\text{NAD}^+ - \text{N}^6 - [\text{N} - (6\text{-aminohexyl}) - \text{acetamide}]$  has not yet been extensively tested in affinity chromatography and its potential as a general ligand remains to be seen. It was however a very efficient adsorbent for the resolution of a mixture of serum albumin, liver alcohol dehydrogenase and lactate dehydrogenase (III).

The method of synthesizing  $\text{NAD}^+ - \text{N}^6 - [\text{N} - (6\text{-aminohexyl}) - \text{acetamide}]$  is generally applicable to adenine nucleotides. The corresponding derivatives of  $\text{NADP}^+$  (31), AMP, ADP and ATP (32) were recently prepared and the polymer-bound derivatives proved to be interesting affinity chromatography ligands as well as promising active cofactors.

### $\text{N}^6$ acyl substituted nucleotides

Whereas  $\text{N}^6$ -alkylated adenine nucleotides are prepared by rather lengthy and complicated routes,  $\text{N}^6$ -acylated nucleotides may be obtained





by quite simple procedures. The acylated compounds are however less stable towards alkali, and even in physiological media a slow breakdown is observed. In some cases the reported stability has been very low (16), but this could probably be attributed to the fact that no proper characterization was carried out, which may have shown that the presumed N<sup>6</sup>-acylated compound also contained ribose-acylated nucleotides, known to be quite unstable (33).

The N<sup>6</sup>-acylated nucleotides are commonly prepared by treating the parent adenine nucleotide with anhydrides. Thus succinic anhydride and NAD<sup>+</sup> gave N<sup>6</sup>-succinyl-NAD<sup>+</sup>, which subsequently was condensed with an amino group-containing carrier, polyethyleneimine, and the resulting soluble immobilized NAD<sup>+</sup>-derivative studied in cofactor experiments (16). The anhydride of carbobenzoxy-6-aminohexanoic acid was reacted with cAMP and, following deprotection, N<sup>6</sup>-(6-amino-hexanoyl)-cAMP was obtained and used as a ligand for the purification of kinases by affinity chromatography (34).

#### S<sup>6</sup> alkyl substituted nucleotides

It has already been mentioned that 6-mercaptapurine riboside phosphate may serve as an intermediate in the preparation of N<sup>6</sup>-(6-aminoethyl)-AMP. 6-Mercaptapurine riboside phosphate has also been utilized in the preparation of immobilized mercapto analogues of AMP, ATP and NAD<sup>+</sup> (17, 35). In one case the immobilization was effected by merely treating bromoacetylated Sepharose with a solution of 6-mercaptapurine riboside phosphate for five days and then quenching residual bromoacetyl groups with mercaptoethanol (17). One less attractive feature associated with this method is that mercapto-nucleotides are expensive and often commercially unavailable.

#### C8 substituted nucleotides

The synthesis of adenine nucleotides provided with a spacer arm attached to the C8 position follows a comparatively straightforward procedure. The parent nucleotide is initially treated with a buffered (pH 4) bromine solution and the formed 8-bromo-nucleotide is isolated in good yield. Attachment of the spacer is then accomplished by heating the bromo-nucleotide with excess 1,6-diaminohexane.



In paper I the synthesis of 8-(6-aminoethyl)-amino-cAMP is briefly described. The corresponding derivatives of AMP (5'), AMP (2'), ADP, ATP and  $\text{NAD}^+$  have since been prepared (19,20,36) and proved to be versatile ligands for kinases and dehydrogenases.

The procedure outlined is not without difficulties when more labile compounds such as the cofactor  $\text{NAD}^+$  are derivatized. Not only must the bromination be carefully controlled to avoid extensive degradation of the coenzyme but also a protective reduction step is necessary before the final displacement reaction with 1,6-diaminohexane in alkaline medium (20). The corresponding derivatives of ADP and ATP may also be prepared by condensation of the nucleotide with phosphate or pyrophosphate (19).

Several adenine nucleotides ( $\text{AMP}$ ,  $\text{NAD}^+$ ,  $\text{NADP}^+$ ) have been immobilized by means of a diazo linkage, supposedly to position C8 which is known to react readily with diazonium salts due to its high electron density. Diazo-bound  $\text{NAD}^+$  has been exploited as an affinity adsorbent (37,38) and also as an active immobilized cofactor (39). The rather bulky benzoyl group which is, of necessity, introduced in close proximity to the ligand, might in some cases adversely affect the ligand - enzyme interaction.

#### Phosphate substituted nucleotides

P-esterified nucleoside di- and triphosphates are also useful as affinity chromatography ligands for dehydrogenases and some kinases. One example is  $\text{p}^1(6\text{-aminoethyl}) \text{p}^2(5'\text{-adenosyl})$  pyrophosphate, prepared by fusing AMP with amino-protected 6-aminoethyl phosphate (19,40,41). p-Aminophenylesters of ATP and dATP were prepared in a similar fashion and the Sepharose-bound ligands were efficient tools for the purification of a ribonucleotide reductase (42).

Direct attachment of nucleotides to polymer supports via the phosphate group is possible by using carbodiimides to promote esterification. Nucleoside mono-, di- and triphosphates as well as oligonucleotides and polynucleotides were thus coupled to cellulose (43). Dextran-bound DNA has also been obtained by this method (44).





Direct attachment of adenine nucleotides according to the cyanogen bromide method has probably been tried by most investigators interested in immobilized nucleotides but often with little success (V,5,37). Successful attempts have also been reported. The terminal phosphate has been suggested as the site of attachment and a suitable mechanism explaining this advanced (45). Positions N<sup>6</sup> (38,46) and ribose (37) have also been proposed as likely reactive sites. In one case a very high incorporation of ATP into Sepharose was claimed, and the preparation used to phosphorylate succinyl-CoA synthetase (46).

The four-component Ugi-reaction was also utilized for the immobilization of various adenine nucleotides (47). When the conditions were chosen to allow participation of the pyrophosphate moiety of NAD<sup>+</sup>, coupling to amino-substituted Sepharose was achieved, giving a coenzymically active derivative.

#### Ribose substituted nucleotides

It was realized quite early that periodate oxidation of the vicinal ribose hydroxyls to yield aldehyde functions would constitute a suitable step in the preparation of immobilized nucleotides. Several nucleosides, mononucleotides and polynucleotides have been treated with periodate and coupled to aminoethyl cellulose by the formation of a Schiff base linkage. The stability of the linkage is increased by subsequent reduction with borohydride (43). An attractive alternative involves the reaction of adipic acid dihydrazide with cyanogen bromide activated Sepharose and the resulting functionalized carrier then mixed with periodate-oxidized nucleotide (48). The ligand is probably bound via a stable hydrazone linkage and furthermore, the linkage between the carrier and the spacer arm lacks the positive charge that ordinary aminoalkyl spacers introduce (14). Derivatives of AMP, ADP, ATP, NAD<sup>+</sup> and NADP<sup>+</sup> have been synthesized by these approaches, although their use as affinity adsorbents and as immobilized cofactors has not yet been exploited to any great extent. The rather drastic alteration of the ribose entity may prohibit proper interaction with some enzymes (49).

Another type of polymer-bound adenine nucleotide linked presumably



through the ribose moiety is presented in paper IV. Activated Sepharose was treated with 6-aminohexanoic acid giving a support functionalized with carboxyl groups. The carboxyl groups were activated with dicyclohexyl carbodiimide in aqueous pyridine and added  $\text{NAD}^+$  was attached. Ultraviolet measurements revealed that the Sepharose-bound  $\text{NAD}^+$  exhibited the same maximum as unbound  $\text{NAD}^+$  (260 nm). This means that the bulk of the nucleotide is not bound through position  $\text{N}^6$ , since  $\text{N}^6$ -acylated adenine nucleotides have maxima at 270 - 275 nm (33,34). Elimination of this possibility leaves ester linkages to the riboses as the most likely means of attachment. It must be remembered, however, that gel-spectra seldom are of sufficient quality to allow precise interpretations and it is therefore reasonable to assume that a presence of  $\text{N}^6$ -acylated nucleotide in an amount less than 10 - 20 % would not be detectable by ultraviolet analysis (9). An experiment involving controlled alkaline hydrolysis would permit quantification of ribose-bound and  $\text{N}^6$ -bound nucleotide, since the former linkage is more susceptible to hydrolytic cleavage than the latter.

The immobilization procedure is rather simple and obviates the need for expensive nucleotide intermediates and has therefore been adapted to most of the common adenine nucleotides (50). Although the resulting polymer-bound derivatives are rather ill-defined, they have been utilized as affinity adsorbents for both model studies and preparative applications.

In paper IV, Sepharose-bound  $\text{NAD}^+$  was tested as a coenzyme for lactate dehydrogenase. The activity was only about 0.2 % compared with the equivalent amount of free  $\text{NAD}^+$ , but at the time it was quite encouraging that immobilized adenine nucleotides could function as coenzymes at all, a fact that so far had not been shown.





## GENERAL DISCUSSION

Polymer-bound adenine nucleotides have hitherto proved to be exceedingly versatile as ligands for affinity chromatography and promising as components in active cofactor applications. It could also be stated that these superlative characteristics especially should be credited to the chemically homogeneous and well-defined preparations. The importance of using chemically well-defined derivatives and specific attachment procedures was recognized early in this laboratory and has since been consistently advocated. The introduction of Sepharose-bound  $N^6$ -(6-aminohexyl)-AMP (I) provided affinity chromatography with a general ligand, which was not only very efficient for enzyme purification, but also due to its defined properties made it possible to predict more accurately the chromatographic behaviour of an enzyme. The recently synthesized  $N^6$ -(6-aminohexyl)-adenosine 2',5'-bisphosphate and  $N^6$ -(6-aminohexyl)-adenosine 3',5'-bisphosphate have not yet been fully exploited as general ligands but should constitute useful complements to  $N^6$ -(6-aminohexyl)-AMP (II).

The original polymer-bound  $NAD^+$  was ill-defined on the basis of homogeneity and unambiguous chemical nature (IV). Despite its limitations it showed that immobilized cofactors could function coenzymically and thus inspired the later development of  $NAD^+-N^6$ -[N-(6-aminohexyl)-acetamide], which when combined with the proper carrier proved a promising adsorbent for affinity chromatography (III) and an efficient active cofactor (V).

The need for defined derivatives and specific attachment procedures will probably be even more accentuated in the future development of immobilized adenine nucleotides or of immobilized biological molecules in general. It is now recognized that the enzyme - immobilized ligand interaction is not only dependent on the nature of the ligand itself, but evidently is also a function of the hydrophobicity of the extension arm, of ionic sites on the polymer backbone and of media compositions and so forth. Future studies may provide means to quantify all relevant parameters, and thus give a rational basis for design of derivatives with the desired properties. Such well-characterized polymer-bound



cofactors, inhibitors and effectors will besides from being superior affinity adsorbents, also find interesting applications in morphological and mechanistic studies of enzymes.

The development of these theoretical aspects will parallel another trend in the field of polymer-bound nucleotides. Several useful adenine nucleotide derivatives are already available and refinements in the methods of their synthesis would allow commercial production at reasonable costs. Large-scale enzyme production by affinity chromatography would then be an attractive alternative to conventional methods and promote a more extended use of enzymes. The combination polymer-bound enzyme - polymer-bound coenzyme, in particular, appears to have a very interesting future, not only in production processes but also in analytical methods and perhaps as components in artificial organs to cure enzyme deficiency diseases and other malfunctions of the body.





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